

PATENT  
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**APPLICATION FOR UNITED STATES LETTERS PATENT  
for  
STARS - A MUSCLE-SPECIFIC ACTIN-BINDING PROTEIN  
by  
Eric Olson  
and  
Akiko Arai**

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## **BACKGROUND OF THE INVENTION**

The government may own rights in the present invention pursuant to grant number RO1HL61544 from the National Institute of Health. The present application claims benefit of 5 priority to U.S. Provisional Serial No. 60/404,706, filed August 20, 2002, the entire contents of which are hereby incorporated by reference.

### **1. Field of the Invention**

The present invention relates generally to the fields of developmental biology and 10 molecular biology. More particularly, it concerns an actin binding protein expressed specifically in striated muscle tissue.

### **2. Description of Related Art**

The actin cytoskeleton influences diverse cellular processes, including motility, mitosis, 15 contractility, cytokinesis, endocytosis and secretion (Burridge and Chrzanowska-Wodnicka, 1996; Schmidt and Hall, 1998; Pantaloni *et al.*, 2001). Actin also participates in numerous transmembrane signaling systems by forming complexes with cell adhesion molecules and receptors (Juliano and Haskill, 1993; Calderwood *et al.*, 2000). In addition, actin has been implicated in the control of gene transcription through its direct association with chromatin 20 remodeling complexes (Rando *et al.*, 2000) and through indirect mechanisms mediated by changes in cytoskeletal actin dynamics (Sotiropoulos *et al.*, 1999) .

Actin exists in monomeric (G-actin) and polymerized (F-actin) forms. The distribution of actin between these two forms is tightly regulated and is influenced by numerous actin-binding proteins which control actin dynamics by severing (*i.e.* ADF/cofilin), cross-linking (*i.e.* 25 actinin, tropomyosin), and capping (*i.e.* tropomodulin at the point ends and capZ at the Z-line) actin (Cooper and Schafer, 2000). Members of the Rho GTPase family regulate cytoskeletal organization by stimulating actin polymerization and stress fiber formation when activated by extracellular signaling (Ridley and Hall, 1992). A number of Rho effector molecules, including 30 Rho kinase/ROCK, mDia and phosphatidylinositol phosphate 5-kinase also participate in cytoskeletal organization (Kaibuchi *et al.*, 1999; Maekawa *et al.*, 1999; Narumiya *et al.*, 1997; Yamamoto *et al.*, 2001; Van Aelst and D'Souza-Schorey, 1997).

Recent studies showed that RhoA signaling stimulates the transcriptional activity of serum response factor (SRF) through a mechanism mediated by changes in actin dynamics (Sotiropoulos *et al.*, 1999; Mack *et al.*, 2001). SRF is a MADS-box transcription factor that regulates serum-inducible and muscle-specific gene expression by binding to a consensus sequence known a CArG box, CC(A/T)6GG (Treisman, 1995a; Treisman, 1995b). The spectrum of genes regulated by SRF is dictated by its association with serum-regulated and muscle-restricted cofactors (Treisman, 1994). In light of the unique and elaborate cytoskeletal organization of striated muscle cells, and the sensitivity of SRF to actin dynamics, it is tempting to speculate that cytoskeletal signals in striated muscle cells might regulate SRF activity in a muscle-specific manner.

### SUMMARY OF THE INVENTION

In one aspect, the present invention provides an isolated STARS polypeptide, in particular a STARS polypeptide comprising an amino acid sequence of SEQ ID NO:2, 4, 6, 8 or 10. By way of illustration, the polynucleotide may have the nucleic acid sequence of SEQ ID NO:1, 3, 5, 7 or 9, or a complement thereof. The polynucleotide may further comprise a promoter operable in eukaryotic cells, for example, a promoter heterologous to the natural sequence of SEQ ID NO:1, 3, 5, 7 or 9. Exemplary promoters include hsp68, SV40, CMV, MKC, GAL4<sub>UAS</sub>, HSV, and β-actin. The promoter may be a tissue specific promoter, such as a cardiac specific promoter.

In another embodiment, there is provided a nucleic acid of 15 to about 2000 base pairs comprising from about 15, 20, 25, 30, 40, 50, 100, 150, 250, 500, 1000, 2000 or more contiguous base pairs of SEQ ID NO:1, 3, 5, 7 or 9, or the complement thereof. Also provided is a peptide comprising 10, 15, 20, 25, 30, 50 or more contiguous amino acids of SEQ ID NO:2, 4, 6, 8 or 10.

In yet another embodiment, there is provided an expression construct comprising a polynucleotide encoding a STARS polypeptide operably linked to a regulatory sequence, for example, a STARS polypeptide having the sequence of SEQ ID NO:2, 4, 6, 8 or 10. In particular embodiments, the polynucleotide within the expression construct is under the control of a tissue specific promoter operable in eukaryotic cells where the promoter may be muscle specific. Exemplary tissue specific promoters include myosin light chain-2 promoter, α actin

promoter, troponin 1 promoter,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger promoter, dystrophin promoter, creatine kinase promoter,  $\alpha 7$  integrin promoter, brain natriuretic peptide promoter,  $\alpha B$ -crystallin/small heat shock protein promoter,  $\alpha$  myosin heavy chain promoter and atrial natriuretic factor promoter. The promoter may be an inducible promoter.

5 The expression construct may be comprised within a viral vector, for example, a retroviral vector, an adenoviral vector, and adeno-associated viral vector, a vaccinia viral vector, a herpesviral vector, a polyoma viral construct or a Sindbis viral vector. The expression construct may further comprise various regulatory sequences, such as, for example, a polyadenylation signal or the like. The expression construct may comprise a one or more 10 additional polynucleotides encoding one or more additional polypeptides, under the control of the same or a different promoter.

In still another embodiment, there is provided a method of screening for modulators of STARS expression comprising (a) providing a cell in which a STARS promoter directs the expression of a polypeptide; (b) contacting said cell with a candidate modulator; and (c) 15 measuring the effect of said candidate modulator on said polypeptide, wherein a difference in expression of said polypeptide, as compared to an untreated cell, indicates that said candidate modulator is a modulator of STARS expression. Measuring may comprise Northern analysis, PCR, RT-PCR, or immunologic detection of STARS (including ELISA and immunohistochemistry). The cell may be located in an animal. The cell type may be a myocyte, 20 or more specifically, a cardiomyocyte. The modulator may increase or decrease expression. The polypeptide may be STARS or a screenable marker polypeptide.

In still yet another embodiment, there is provided a method of screening for modulators of STARS actin binding activity comprising (a) providing an active STARS preparation; (b) contacting said STARS preparation with a candidate modulator; and (c) measuring the actin 25 binding activity of said STARS preparation, wherein a difference in actin binding activity of said STARS preparation, as compared to an untreated STARS preparation, indicates that said candidate modulator is a modulator of STARS actin binding activity. The screening may be performed in a cell free assay or in a cell. The binding may be determined by chromatographic separation or electrophoretic separation.

30 Further embodiments include a method of screening for an inhibitor of STARS-induced transcription comprising (a) providing a cell that expresses STARS and contains a STARS-

regulated promoter linked to an indicator gene; (b) contacting said cell with a candidate modulator; and (c) measuring the effect of said candidate modulator on expression of said indicator gene, wherein a difference in expression of said polypeptide, as compared to an untreated cell, indicates that said candidate modulator is a modulator of STARS-induced transactivation. The cell may be a myocyte, for example, a cardiomyocyte. The promoter may be an *SM22* promoter. The indicator gene may encode luciferase,  $\beta$ -galactosidase, CAT, or green fluorescent protein.

Also provided is a method of producing a STARS polypeptide in a cell comprising (a) transforming a cell with an expression cassette comprising a nucleic acid encoding STARS under the control of a promoter active in said cell; (b) culturing said cell under conditions suitable for expression of STARS. The cell may be, for example, a cardiomyocyte or a fibroblast, such as a cardiac fibroblast. The cell may be located in an animal. The transforming step may comprise infection with a viral vector. The transforming step may also comprise contacting the cell with a liposome comprising the expression cassette, electroporation, calcium phosphate precipitation, or protoplast fusion. The cell may be a prokaryotic or eukaryotic cell. The method may further comprise the step of purifying said STARS polypeptide away from other cellular components.

In other embodiments, there are provided a non-human transgenic animal comprising a selectable or screenable marker protein under the control of a STARS promoter; a non-human transgenic animal comprising a STARS encoding nucleic acid under the control of an inducible promoter; a non-human transgenic animal comprising a STARS encoding nucleic acid under the control of a constitutive promoter; and a non-human transgenic animal lacking at least one STARS allele, or both.

In other embodiments, there are provided a method of inhibiting STARS activity comprising contacting a cell expressing STARS with a compound that inhibits STARS activity. The compound may be a nucleic acid antisense to a STARS regulatory or coding region, a ribozyme that selectively cleaves a STARS transcript, a small molecule inhibitor, or a single chain antibody that binds immunologically to STARS.

In yet other embodiments, methods of treating cardiac hypertrophy and dilated cardiomyopathy comprising decreasing STARS activity in heart cells of a subject are provided. In one aspect, STARS activity is decreased by delivering an expression vector comprising a polynucleotide encoding an antisense STARS construct, a STARS ribozyme or an anti-STARS

single-chain antibody to said subject. The expression vector may be a non-viral or a viral vector. The viral vector may be an adenoviral construct, a retroviral construct, an adeno-associated viral construct, a herpesviral construct, a vaccinia viral construct, a polyoma viral construct or a Sindbis viral vector. The viral vector may be a replication-defective adenovirus.

5 By way of illustration, the step of delivering the expression construct may comprise introducing a viral vector comprising the nucleic acid into the heart of the mammal by direct injection into the heart tissue. The step of delivering the expression construct may also comprise introducing the expression construct into the lumen of at least one vessel that supplies blood to the heart. Delivering the expression construct may further comprise administering a second anti-  
10 hypertrophic drug, or decreasing STARS activity in hearts cells of a subject.

In additional embodiments, there are provided:

a method of producing a modulator of STARS expression comprising: (a) providing a  
15 cell in which a STARS promoter directs the expression of a polypeptide; (b) contacting said cell with a candidate modulator; (c) measuring the effect of said candidate modulator on said polypeptide, wherein a difference in expression of said polypeptide, as compared to an untreated cell, indicates that said candidate modulator is a modulator of STARS expression; and (d) producing said modulator;

a method of producing a modulator of STARS actin binding activity comprising: (a) providing an active STARS preparation; (b) contacting said STARS preparation with a candidate modulator; (c) measuring the actin binding activity of said STARS preparation, wherein a difference in actin binding activity of said STARS preparation, as compared to an untreated STARS preparation, indicates that said candidate modulator is a modulator of STARS actin binding activity; and (d) producing said modulator;  
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a modulator of STARS expression identified according to the method comprising: (a) providing a cell in which a STARS promoter directs the expression of a polypeptide; (b) contacting said cell with a candidate modulator; and (c) measuring the effect of said candidate modulator on said polypeptide, wherein a difference in expression of said polypeptide, as compared to an untreated cell, indicates that said candidate modulator is a modulator of STARS expression;  
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5 a modulator of STARS actin binding activity identified according to the method comprising: (a) providing a STARS preparation; (b) contacting said STARS preparation with a candidate modulator; and (c) measuring the actin binding activity of said STARS preparation, wherein a difference in actin binding activity of said STARS preparation, as compared to an untreated STARS preparation, indicates that said candidate modulator is a modulator of STARS actin binding activity;

10 There also are provided an antibody that binds immunologically to STARS, a polyclonal antibody preparation of antibodies that bind immunologically to STARS, and a hybridoma cell that produces a monoclonal antibody that binds immunologically to STARS.

15 In other embodiments, there are provided a method of treating myocardial infarct comprising decreasing STARS activity in heart cells of a subject; a method of preventing cardiac hypertrophy and dilated cardiomyopathy comprising decreasing STARS activity in heart cells of a subject; a method of inhibiting progression of cardiac hypertrophy comprising decreasing STARS activity in heart cells of a subject; a method of treating heart failure comprising decreasing STARS activity in heart cells of a subject; a method of inhibiting progression of heart failure comprising decreasing STARS activity in heart cells of a subject; a method of increasing exercise tolerance in a subject with heart failure or cardiac hypertrophy comprising decreasing STARS activity in heart cells of a subject; a method of reducing hospitalization in a subject with 20 heart failure or cardiac hypertrophy comprising decreasing STARS activity in heart cells of a subject; a method of improving quality of life in a subject with heart failure or cardiac hypertrophy comprising decreasing STARS activity in heart cells of a subject; a method of decreasing morbidity in a subject with heart failure or cardiac hypertrophy comprising decreasing STARS activity in heart cells of a subject; and a method of decreasing mortality in a 25 subject with heart failure or cardiac hypertrophy comprising decreasing STARS in heart cells of a subject.

#### BRIEF DESCRIPTION OF THE DRAWINGS

30 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by

reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1 - Deduced amino acid sequence and homology of STARS to other predicted proteins.**

5 Deduced amino acid sequence of mouse STARS (Accession numbers AF504061 and AAM28877) and homology with sequences from other species, including human (AF503617 and AAM27268).

**FIG. 2 - Expression of STARS in cardiac and skeletal muscle.** FIG. 2A: *In situ* hybridization

10 shows the specific expression of STARS transcripts in the heart tube of E8.75 mouse embryo h, head; ht, heart. FIG. 2B: Northern blot analysis of STARS transcripts in adult mouse and human tissues detected with Clontech multi tissue blots. FIG. 2C: Northern blot analysis of STARS and GAPDH transcripts during differentiation of the C2 muscle cell line. At day 0, myoblasts were maintained in growth medium and were then transferred to differentiation medium for the  
15 indicated number of days. FIG. 2D: Northern analysis of STARS and GAPDH transcripts in hearts from wild-type (WT) and  $\alpha$ -MHC-calcineurin transgenic (Tg) mice at 3 and 6 months of age. Twenty  $\mu$ g total RNA was applied to each lane in FIG. 2C and FIG. 2D. Positions of 18S and 28S RNA are indicated.

20 **FIG. 3 - Identification of STARS as a sarcomeric protein.** FIG. 3A: Western blot analysis using

anti-STARS antibody detected endogenous STARS protein in adult mouse heart lysate and in COS cells transfected with STARS expression vector. FIG. 3B: Localization of STARS to the actin thin-filament and stress fibers in cardiomyocytes. Rat neonatal cardiomyocytes were infected with GFP adenovirus (a, b) or STARS adenovirus (c-h). Anti STARS antibody does not

25 detect GFP protein nor endogenous STARS (a). Overexpressed STARS is stained with anti-STARS antibody followed by fluoresceine anti-rabbit IgG secondary antibody (c, f). Z- band is stained with anti  $\alpha$ -actinin antibody followed by Texas red conjugated secondary antibody (b, d). STARS was localized in the sarcomere (c and f), but not at the Z-band, as demonstrated by the lack of overlap with  $\alpha$ -actinin (d, e). Phalloidin-TRITC shows the thin filaments (g). STARS  
30 superimposes with the thin filament, but not at Z-lband (h). Bars: 10  $\mu$ m (e, a-e in the same magnification), 5  $\mu$ m (h)

**FIG. 4 - Deletion mapping of the actin-binding domain of STARS.** FIG. 4A: COS cells were transiently transfected with an expression vector encoding STARS (wt) or deletion mutants with an N-terminal Myc-tag. The subcellular distribution of STARS was determined by immunofluorescence using fluoresceine anti-mouse IgG secondary antibody (c, f, i and l). Cells were also stained with Phalloidin-TRITC to visualize F- actin (a, b, d, g, j and m). F-actin and stress fibers were in COS cells transfected with an empty vector (a, b). STARS (wt), shows the exact same pattern of phalloidin staining; strongly associates with F-actin and induces extremely thick bundles (c, d, e). Numerous tumble stress fibers are also induced. (f, g, h). Homologous region C142 induces actin bundles (i, j, k). In contrast, homologous region deletion mutant C96 just associates with actin stress fibers (l, m, n). Bar indicates 20  $\mu$ m. FIG. 4B: COS cells were transiently transfected with an expression vector encoding STARS or the indicated deletion mutants with Myc-epitope tags. Cell extracts were then immunoprecipitated with a rabbit polyclonal anti-Myc antibody and immunoprecipitates were analyzed by Western blot using anti-actin monoclonal antibody (top) or anti-Myc monoclonal antibody (bottom).

**FIG. 5 - Summary of STARS deletion mutants.** STARS (wt) and all deletion mutants associates with actin stress fibers in immunocytochemistry, Wild type STARS and deletion mutants (C142, Del234-247, Del248-262 and Del263-279) have an ability to induce actin bundles and activate SRF transcription.

**FIG. 6 - STARS stimulates SRF activity through a Rho-dependent mechanism.** FIG. 6A: COS and F9 cells were transiently transfected with an *SM22*-luciferase reporter or the same reporter containing mutations in the CArG-boxes within the *SM22* promoter (mut*SM22*), and an STARS expression vector. FIG. 6B: COS cells were transiently transfected with the indicated reporter plasmids with and without an STARS expression plasmid. FIG. 6C: COS cells were transiently transfected with *SM22*-luciferase and expression plasmids encoding the indicated STARS mutants. FIG. 6D: COS cells were transiently transfected with *SM22*-luciferase in the presence and absence of STARS and the indicated agents. FIG. 6E: COS cells were transiently transfected with *SM22*-luciferase and a STARS or RhoA (L63) expression vector. FIG. 6F: COS cells were transiently transfected with *SM22*-luciferase and a STARS expression vector in the

presence of Y-27632 or a C3 expression vector. Luciferase activity was determined on cell extracts and is expressed as the level of activity relative to that in cells transfected with the indicated reporters without STARS.

5 **FIG. 7** – A model for the involvement of STARS in actin dynamics and signaling to SRF. The distribution of actin between the polymerized (F actin) and monomeri (G actin) states is determined by actin treadmilling. Actin monomers are added to the plus end and are removed from the minus end of actin filaments. STARS promotes the formation of F actin and activates Rho, which also promotes actin polymerization. Rho activates ROCK and other effectors  
10 leading to the stimulation of SRF activity.

#### DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Heart disease and its manifestations, including coronary artery disease, myocardial infarction, congestive heart failure and cardiac hypertrophy, presents a major health risk in the United States today. The cost to diagnose, treat and support patients suffering from these diseases is well into the billions of dollars. Two particularly severe manifestations of heart disease are myocardial infarction and cardiac hypertrophy. With respect to myocardial infarction, typically an acute thrombotic coronary occlusion occurs in a coronary artery as a result of atherosclerosis and causes myocardial cell death. Because cardiomyocytes, the heart muscle cells, are terminally differentiated and generally incapable of cell division, they are generally replaced by scar tissue when they die during the course of an acute myocardial infarction. Scar tissue is not contractile, fails to contribute to cardiac function, and often plays a detrimental role in heart function by expanding during cardiac contraction, or by increasing the size and effective radius of the ventricle, for example, becoming hypertrophic. With respect to cardiac hypertrophy, one theory regards this as a disease that resembles aberrant development and, as such, raises the question of whether developmental signals in the heart can contribute to hypertrophic disease.

The inventors have described herein a novel actin-binding protein designated as STARS  
30 (striated muscle activator of Rho signaling), which is expressed specifically in cardiac and skeletal muscle cells and is upregulated in response to calcineurin signaling during cardiac

hypertrophy. STARS is identified in a differential cDNA screen for unknown genes expressed in the early embryonic heart. It is localized to the thin filament of the sarcomere and to actin stress fibers where it promotes actin bundling. STARS stimulates the transcriptional activity of SRF through a mechanism that requires actin bundling and Rho kinase activation. STARS provides a 5 mechanism for selectively enhancing the transcriptional activity of SRF in muscle cells and for linking changes in actin dynamics to gene transcription. These findings indicate that STARS acts as a muscle-specific transducer of cytoskeletal signals that stimulate SRF activity.

### I. STARS Peptides and Polypeptides

10 STARS is a novel and evolutionarily conserved actin-binding/bundling protein expressed specifically in striated muscle. STARS localizes to the thin filament of the sarcomere and to actin stress fibers. Stabilization of the actin cytoskeleton by STARS stimulates SRF-dependent transcription through a mechanism that involves RhoA signaling.

15 STARS contains no recognizable protein motifs and represents a new type of actin-binding protein. Deletion mutants of STARS indicate that there are multiple, nonoverlapping regions of the protein that can mediate association with actin stress fibers *in vivo*. However, only the conserved carboxy-terminal domain bundles F-actin and binds actin with sufficient affinity to be detect in co-immunoprecipitation assays. The high conservation of this region between vertebrate and invertebrate proteins indicates that this activity has been evolutionarily conserved. 20 It should also be noted that STARS associates with actin of the thin-filament, but not the Z-line. This raises the possibility that the STARS-binding region of actin may be masked on the Z-line, possibly because of numerous other actin-binding proteins localized to this structure (Bang *et al.*, 2001).

25 STARS expression is initiated in cardiac and skeletal muscle during the period of myofibrillogenesis when the myofibrillar components become assembled into the functional sarcomere (Ehler *et al.*, 1999; Gregorio and Antin, 2000). Considering the timing of its expression and its actin-binding properties, STARS may participate in sarcomere assembly by inducing actin polymerization and cross-linking during striated muscle development.

30 Consistent with recent studies implicating actin dynamics in the control of SRF dependent transcription (Sotiropoulos *et al.*, 1999; Wei *et al.*, 2001; Mack *et al.*, 2001), STARS stimulates the activity of SRF. The conserved carboxy-terminal region of STARS is both

necessary and sufficient for SRF activation and actin bundling; the correlation between these activities argues that the effects of STARS on SRF are coupled to its effects on the cytoskeleton. Stimulation of SRF activity is observed with native muscle promoters, as well as with an artificial promoter containing multimerized CArG-boxes. However, STARS does not activate all  
5 SRF-dependent promoters equally, which indicates that additional promoter-specific transcription factors modulate the responsiveness of SRF to STARS-dependent signaling.

In addition to an entire STARS molecule, the present invention also relates to fragments of the polypeptides that may or may not retain several of the functions described below. Fragments, including the N-terminus of the molecule, may be generated by genetic engineering  
10 of translation stop sites within the coding region (discussed below). Alternatively, treatment of the STARS with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, and 10 of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200, 300, 400 or more amino acids  
15 in length. These fragments may be purified according to known methods, such as precipitation (e.g., ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

20 **A. Variants of STARS**

Amino acid sequence variants of the polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein that are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking  
25 secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of an immunoreactive epitope or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for another at one  
30 or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or

properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. Table 1 shows the codons that encode particular amino acids.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are 5 within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

10 As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine \*-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

15 It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

20 As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take several of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, 25 leucine and isoleucine.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure (Johnson *et al.*, 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in 30 such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These

principles may be used, in conjunction with the principles outline above, to engineer second generation molecules having many of the natural properties of STARS, but with altered and even improved characteristics.

5           **B. Domain Switching**

Domain switching involves the generation of chimeric molecules using different but, in this case, related polypeptides. These molecules may have additional value in that these "chimeras" can be distinguished from natural molecules, while possibly providing the same function. For example, any of the various homologs from other species provide suitable  
10 candidates for domain switching experiments.

**C. Fusion Proteins**

A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion  
15 of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include  
20 linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

**D. Purification of Proteins**

It will be desirable to purify STARS or variants thereof. Protein purification techniques  
25 are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a  
30 pure peptide are ion-exchange chromatography, exclusion chromatography, polyacrylamide gel

electrophoresis, and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to 5 specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by 10 cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to 15 purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant 20 extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in 25 accord with the present invention is discussed below.

#### **E. Synthetic Peptides**

The present invention also describes smaller STARS-related peptides for use in various 30 embodiments of the present invention. Because of their relatively small size, the peptides of the invention can also be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be

used in accordance with known protocols. See, for example, Stewart and Young (1984); Tam *et al.* (1983); Merrifield (1986); and Barany and Merrifield (1979), each incorporated herein by reference. Short peptide sequences, or libraries of overlapping peptides, usually from about 6 up to about 35 to 50 amino acids, which correspond to the selected regions described herein, can be  
5 readily synthesized and then screened in screening assays designed to identify reactive peptides. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

10           **F.     Antigen Compositions**

The present invention also provides for the use of STARS proteins or peptides as antigens for the immunization of animals relating to the production of antibodies. It is envisioned that STARS, or portions thereof, will be coupled, bonded, bound, conjugated or chemically-linked to one or more agents via linkers, polylinkers or derivatized amino acids. This may be performed  
15 such that a bispecific or multivalent composition or vaccine is produced. It is further envisioned that the methods used in the preparation of these compositions will be familiar to those of skill in the art and should be suitable for administration to animals, *i.e.*, pharmaceutically acceptable. Preferred agents as the carriers are keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).

20

**II.    Nucleic Acids**

The present invention also provides, in another embodiment, genes encoding STARS. Genes for mouse, human, zebrafish, *Drosophila* or *c. elegans* have been identified. See, for example, SEQ ID NOS: 1, 3, 5, 7 and 9 respectively. The present invention is not limited in  
25 scope to these genes, however, as one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in these and various other species (*e.g.*, rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a “STARS gene” may contain a variety of  
30 different bases and yet still produce a corresponding polypeptide that is functionally

indistinguishable, and in some cases structurally, from the human and mouse genes disclosed herein.

Similarly, any reference to a nucleic acid should be read as encompassing a host cell containing that nucleic acid and, in some cases, capable of expressing the product of that nucleic acid. In addition to therapeutic considerations, cells expressing nucleic acids of the present invention may prove useful in the context of screening for agents that induce, repress, inhibit, augment, interfere with, block, abrogate, stimulate or enhance the activity of STARS.

#### A. Nucleic Acids Encoding STARS

Nucleic acids according to the present invention may encode an entire STARS gene, a domain of STARS, or any other fragment of STARS as set forth herein. The nucleic acid may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In preferred embodiments, however, the nucleic acid would comprise complementary DNA (cDNA). Also contemplated is a cDNA plus a natural intron or an intron derived from another gene; such engineered molecules are sometime referred to as "mini-genes." At a minimum, these and other nucleic acids of the present invention may be used as molecular weight standards in, for example, gel electrophoresis.

The term "cDNA" is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic, non- or partially-processed RNA template, is that the cDNA primarily contains coding sequences of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, such as where the non-coding regions are required for optimal expression or where non-coding regions such as introns are to be targeted in an antisense strategy.

It also is contemplated that a given STARS from a given species may be represented by natural variants that have slightly different nucleic acid sequences but, nonetheless, encode the same protein (see Table 1 below).

As used in this application, the term "a nucleic acid encoding a STARS" refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. In preferred embodiments, the invention concerns a nucleic acid sequence essentially as set forth in SEQ ID NOS: 1, 3, 5, 7 or 9 (mouse, human, zebrafish, *Drosophila* or *c. elegans* respectively). The term "as set forth in SEQ ID NOS: 1 or 3, 5, 7 or 9" means that the nucleic acid sequence substantially corresponds to a

portion of SEQ ID NO:1 or 3, 5, 7 or 9. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine (Table 1, below), and also refers to codons that encode biologically equivalent amino acids, as discussed in the following pages.

5

TABLE 1

Amino Acids			Codons				
Alanine	Ala	A	GCA	GCC	GCG	GCU	
Cysteine	Cys	C	UGC	UGU			
Aspartic acid	Asp	D	GAC	GAU			
Glutamic acid	Glu	E	GAA	GAG			
Phenylalanine	Phe	F	UUC	UUU			
Glycine	Gly	G	GGA	GGC	GGG	GGU	
Histidine	His	H	CAC	CAU			
Isoleucine	Ile	I	AUA	AUC	AUU		
Lysine	Lys	K	AAA	AAG			
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG
Methionine	Met	M	AUG				
Asparagine	Asn	N	AAC	AAU			
Proline	Pro	P	CCA	CCC	CCG	CCU	
Glutamine	Gln	Q	CAA	CAG			
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG
Threonine	Thr	T	ACA	ACC	ACG	ACU	
Valine	Val	V	GUU	GUC	GUG	GUU	
Tryptophan	Trp	W	UGG				
Tyrosine	Tyr	Y	UAC	UAU			

Allowing for the degeneracy of the genetic code, sequences that have at least about 50%, usually at 10 least about 60%, more usually about 70%, most usually about 80%, preferably at least about 90% and most preferably about 95% of nucleotides that are identical to the nucleotides of SEQ ID NOS:1 or 3, 5, 7 or 9 are contemplated. Sequences that are essentially the same as those set forth in SEQ ID NOS:1, 3, 5, 7 or 9 may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NOS:1, 3, 5, 7 or 9 under standard 15 conditions.

The DNA segments of the present invention include those encoding biologically functional equivalent STARS proteins and peptides, as described above. Such sequences may arise as a consequence of codon redundancy and amino acid functional equivalency that are known to occur

naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced 5 through the application of site-directed mutagenesis techniques or may be introduced randomly and screened later for the desired function, as described below.

#### **B. Oligonucleotide Probes and Primers**

Naturally, the present invention also encompasses DNA segments that are complementary, 10 or essentially complementary, to the sequence set forth in SEQ ID NOS:1, 3, 5, 7 or 9. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic 15 acid segment of SEQ ID NOS:1, 3, 5, 7 or 9 under relatively stringent conditions such as those described herein. Such sequences may encode entire STARS proteins or functional or non-functional fragments thereof.

Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique 20 target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs 25 will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, 3000 or 5000 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

Suitable hybridization conditions will be well known to those of skill in the art. In certain 30 applications, for example, substitution of amino acids by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur

even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low 5 stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, at temperatures between 10 approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 µM MgCl<sub>2</sub>, at temperatures ranging from approximately 40°C to about 72°C. Formamide and SDS also may be used to alter the hybridization conditions.

One method of using probes and primers of the present invention is in the search for genes 15 related to STARS or, more particularly, homologs of STARS from other species. Normally, the target DNA will be a genomic or cDNA library, although screening may involve analysis of RNA molecules. By varying the stringency of hybridization, and the region of the probe, different degrees of homology may be discovered.

Another way of exploiting probes and primers of the present invention is in site-directed, 20 or site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis 25 allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the 30 junction of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely 5 employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double-stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired 10 mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand 15 bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as 20 there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

### C. Antisense Constructs

25 Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine 30 (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of

less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced 5 into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

10 Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been 15 observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is 20 affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are 25 completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (e.g., ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target 30 sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the 5 construct and, therefore, would be used for the rest of the sequence.

#### **D. Ribozymes**

Although proteins traditionally have been used for catalysis of nucleic acids, another 10 class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic 15 domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and 15 Shub, 1992). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific 20 cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a 25 sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cell lines to which 25 they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

#### **E. Vectors for Cloning, Gene Transfer and Expression**

30 Within certain embodiments expression vectors are employed to express a STARS polypeptide product, which can then be purified and, for example, be used to vaccinate animals to

generate antisera or monoclonal antibody with which further studies may be conducted. In other embodiments, the expression vectors are used in gene therapy. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes 5 of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

10

(i) Regulatory Elements

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be 15 translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the 20 synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules 25 that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites 30 for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the STARSt site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the STARSt site itself 5 helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the STARSt site, although a number of promoters have recently been shown to contain functional elements downstream of the STARSt site as well. The spacing between promoter elements frequently is flexible, so that 10 promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene 15 promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient 20 for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 2 and 3 list several regulatory elements that 25 may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. 30 That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular 5 orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 2 and Table 3). Additionally, any other 10 promoter/enhancer combination (for example, as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 2	
Promoter and/or Enhancer	
Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl <i>et al.</i> , 1985; Atchinson <i>et al.</i> , 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen <i>et al.</i> , 1983; Picard <i>et al.</i> , 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto <i>et al.</i> , 1989; Redondo <i>et al.</i> ; 1990
HLA DQ $\alpha$ and/or DQ $\beta$	Sullivan <i>et al.</i> , 1987
$\beta$ -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn <i>et al.</i> , 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DRA	Sherman <i>et al.</i> , 1989
$\beta$ -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989

**TABLE 2**  
Promoter and/or Enhancer

Promoter/Enhancer	References
Muscle Creatine Kinase (MCK)	Jaynes <i>et al.</i> , 1988; Horlick <i>et al.</i> , 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Ornitz <i>et al.</i> , 1987
Metallothionein (MTII)	Karin <i>et al.</i> , 1987; Culotta <i>et al.</i> , 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987a
Albumin	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
$\alpha$ -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere <i>et al.</i> , 1989
t-Globin	Bodine <i>et al.</i> , 1987; Perez-Stable <i>et al.</i> , 1990
$\beta$ -Globin	Trudel <i>et al.</i> , 1987
c-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990
$\alpha_1$ -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse and/or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor (PDGF)	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990

**TABLE 2**  
Promoter and/or Enhancer

Promoter/Enhancer	References
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleigh <i>et al.</i> , 1985; Firak <i>et al.</i> , 1986; Herr <i>et al.</i> , 1986; Imbra <i>et al.</i> , 1986; Kadesch <i>et al.</i> , 1986; Wang <i>et al.</i> , 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber <i>et al.</i> , 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler <i>et al.</i> , 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander <i>et al.</i> , 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Choi <i>et al.</i> , 1988; Reisman <i>et al.</i> , 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and/or Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky <i>et al.</i> , 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens <i>et al.</i> , 1987; Glue <i>et al.</i> , 1988
Hepatitis B Virus	Bulla <i>et al.</i> , 1986; Jameel <i>et al.</i> , 1986; Shaul <i>et al.</i> , 1987; Spandau <i>et al.</i> , 1988; Vannice <i>et al.</i> , 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber <i>et al.</i> , 1988; Jakobovits <i>et al.</i> , 1988; Feng <i>et al.</i> , 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp <i>et al.</i> , 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus (CMV)	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking <i>et al.</i> , 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

**TABLE 3**  
Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger <i>et al.</i> , 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1991; Lee <i>et al.</i> , 1981; Majors <i>et al.</i> , 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
$\beta$ -Interferon	poly(rI)x poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	E1A	Imperiale <i>et al.</i> , 1984
Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug <i>et al.</i> , 1988
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
$\alpha$ -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2 $\kappa$ b	Interferon	Blanar <i>et al.</i> , 1989
HSP70	E1A, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq <i>et al.</i> , 1989
Tumor Necrosis Factor	PMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone $\alpha$ Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

Of particular interest are muscle specific promoters, and more particularly, cardiac specific promoters. These include the myosin light chain-2 promoter (Franz *et al.*, 1994; Kelly *et al.*, 1995), the  $\alpha$  actin promoter (Moss *et al.*, 1996), the troponin 1 promoter (Bhavsar *et al.*, 1996); the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger promoter (Barnes *et al.*, 1997), the dystrophin promoter (Kimura *et al.*, 1997), the creatine kinase promoter (Ritchie, M.E., 1996), the  $\alpha 7$  integrin promoter (Ziober & Kramer, 1996), the brain natriuretic peptide promoter (LaPointe *et al.*, 1996), the  $\alpha$  B-crystallin/small heat shock protein promoter (Gopal-Srivastava, R., 1995), and  $\alpha$  myosin heavy chain promoter (Yamauchi-Takihara *et al.*, 1989) and the ANF promoter (LaPointe *et al.*, 1988).

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

10 (ii) Selectable Markers

In certain embodiments of the invention, the cells contain nucleic acid constructs of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

(iii) Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and 5 begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornaovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating 10 polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular 15 or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

(iv) Delivery of Expression Constructs

20 There are a number of ways in which expression constructs may be introduced into cells. In certain embodiments of the invention, a vector (also referred to herein as a gene delivery vector) is employed to deliver the expression construct. By way of illustration, in some embodiments, the vector comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into 25 host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene delivery vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986). Generally, these 30 have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. They can accommodate only up to 8 kb of foreign genetic material but can be readily introduced

in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986). Where viral vectors are employed to deliver the gene or genes of interest, it is generally preferred that they be replication-defective, for example as known to those of skill in the art and as described further herein below.

5 One of the preferred methods for *in vivo* delivery of expression constructs involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express a polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

10 In preferred embodiments, the expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can  
15 replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage and are able to infect non-dividing cells such as, for example, cardiomyocytes. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in  
20 humans.

Adenovirus is particularly suitable for use as a gene delivery vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions  
25 of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The  
30 products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter

(MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is important to minimize this possibility by, for example, reducing or eliminating adenoviral sequence overlaps within the system and/or to isolate a single clone of virus from an individual plaque and examine its genomic structure.

10 Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA  
15 in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of such adenovirus vectors is about 7.5 kb, or about 15% of the total length of the vector. Additionally, modified  
20 adenoviral vectors are now available which have an even greater capacity to carry foreign DNA.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey  
25 embryonic mesenchymal or epithelial cells. As stated above, a preferred helper cell line is 293.

Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format,  
30 Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer

flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, 5 following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be selected from any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is a preferred 10 STARSting material for obtaining a replication-defective adenovirus vector for use in the present invention. This is, in part, because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, a preferred adenoviral vector according to the present invention lacks an 15 adenovirus E1 region and thus, is replication. Typically, it is most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. Further, other adenoviral sequences may be deleted and/or inactivated in addition to or in lieu of the E1 region. For example, the E2 and E4 regions 20 are both necessary for adenoviral replication and thus may be modified to render an adenovirus vector replication-defective, in which case a helper cell line or helper virus complex may be employed to provide such deleted/inactivated genes *in trans*. The polynucleotide encoding the gene of interest may alternatively be inserted in lieu of a deleted E3 region such as in E3 replacement vectors as described by Karlsson *et al.* (1986), or in a deleted E4 region where a 25 helper cell line or helper virus complements the E4 defect. Other modifications are known to those of skill in the art and are likewise contemplated herein.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*,  $10^9$ - $10^{12}$  plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into 30 the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of

vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies indicated that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include administration via intracoronary catheter into one or more coronary arteries of the heart (Hammond, *et al.*, U.S. Patents 5,792,453 and 6,100,242) trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and

Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

5 A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

10 A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

15 There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the 20 potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

25 Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; 30 Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This indicated that large portions of 5 the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.*, recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing 10 high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of 15 certain disease states. In general, viral vectors accomplish delivery of the expression construct by infecting the target cells of interest. Alternatively to incorporating the expression construct into the genome of a viral vector, the expression construct may be encapsidated in the infectious viral particle.

Several non-viral gene delivery vectors for the transfer of expression constructs into 20 mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer 25 *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding 30 the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene

replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the 5 host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In yet another embodiment of the invention, the expression vector may simply consist of naked recombinant DNA or plasmids comprising the expression construct. Transfer of the construct may be performed by any of the methods mentioned above which physically or 10 chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate- 15 precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment of the invention, transferring of a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to 20 accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as 25 tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular gene may be delivered via this method 30 and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome, another non-viral gene delivery vector. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously 5 when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has 10 been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a 15 hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the 20 liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a 25 particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell 30 receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid

(ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0 273 085).

5 In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type by any number of receptor-ligand systems with 10 or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid into cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used 15 as targeting moieties.

15 In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of 20 cells and tissues.

### III. Generating Antibodies Reactive With STARS

In another aspect, the present invention contemplates an antibody that is immunoreactive with a STARS molecule of the present invention, or any portion thereof. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal 25 antibody. Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an 30 animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats,

hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

10 It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to STARS-related antigen epitopes. Additionally, it is proposed that monoclonal antibodies specific to the particular STARS of different species may 15 be utilized in other useful applications

In general, both polyclonal and monoclonal antibodies against STARS may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding other STARS. They may also be used in inhibition studies to analyze the effects of STARS related peptides in cells or animals. STARS antibodies will also 20 be useful in immunolocalization studies to analyze the distribution of STARS during various cellular events, for example, to determine the cellular or tissue-specific distribution of STARS polypeptides under different points in the cell cycle. A particularly useful application of such antibodies is in purifying native or recombinant STARS, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in 25 the art in light of the present disclosure.

Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988; incorporated herein by reference). More specific examples of monoclonal antibody preparation are given in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is 30 often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole

limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-  
5 biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*),  
10 incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by  
15 sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

20 MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified STARS protein, polypeptide or peptide or cell expressing high levels of STARS. The immunizing composition is administered in a manner effective to  
25 stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies,  
30 specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood

sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the 5 spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with 10 cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures 15 preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the 15 art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one 20 may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful 25 in connection with cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and 20 myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using 25 polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around  $1 \times 10^{-6}$  to 30  $1 \times 10^{-8}$ . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine.

Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

5 The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in  
10 the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid,  
15 such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the  
20 hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*,  
25 where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

#### **IV. Diagnosing and Treating Defects in STARS**

The inventors believe that STARS plays an important role in the development of cardiac tissue and, further, in the mechanisms of heart disease. Thus, in another embodiment, there are provided methods for diagnosing defects in STARS expression and function. More specifically, 5 point mutations, deletions, insertions or regulatory perturbations relating to STARS, as well as increases or decrease in levels of expression, may be assessed using standard technologies, as described below.

##### **A. Genetic Diagnosis**

10 One embodiment of the instant invention comprises a method for detecting variation in the expression of STARS. This may comprise determining the level of STARS or determining specific alterations in the expressed product.

15 A suitable biological sample can be any tissue or fluid. Various embodiments include cells of the skin, muscle, facia, brain, prostate, breast, endometrium, lung, head & neck, pancreas, small intestine, blood cells, liver, testes, ovaries, colon, skin, stomach, esophagus, spleen, lymph node, bone marrow or kidney. Other embodiments include fluid samples such as peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

20 Nucleic acid used is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

25 Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals 30 (Affymax Technology; Bellus, 1994).

Various types of defects may be identified by the present methods. Thus, "alterations" should be read as including deletions, insertions, point mutations and duplications. Point mutations result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those occurring in non-germline tissues. Germ-line mutations can occur in any 5 tissue and are inherited. Mutations in and outside the coding region also may affect the amount of STARS produced, both by altering the transcription of the gene or in destabilizing or otherwise altering the processing of either the transcript (mRNA) or protein.

It is contemplated that other mutations in the STARS genes may be identified in accordance with the present invention. A variety of different assays are contemplated in this 10 regard, including but not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA), RNase protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR<sup>TM</sup>-SSCP.

15 (i) Primers and Probes

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded 20 form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable of priming, are designed to bind to the target DNA or RNA and need not be used in an amplification process.

In preferred embodiments, the probes or primers are labeled with radioactive species (<sup>32</sup>P, <sup>14</sup>C, <sup>35</sup>S, <sup>3</sup>H, or other label), with a fluorophore (rhodamine, fluorescein) or a chemiluminescent 25 (luciferase).

(ii) Template Dependent Amplification Methods

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the 30 polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patent Nos.

4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990, each of which is incorporated herein by reference in its entirety.

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside 5 triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the 10 marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are 15 described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe 20 pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

25 Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, (1989), incorporated herein by reference in its entirety.

(iii) Southern/Northern Blotting

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting or RNA species.

Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by “blotting” on to the filter.

10 Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

#### (iv) Separation Methods

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See 20 Sambrook *et al.*, 1989.

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

(v) Detection Methods

Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be

exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified 5 marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well known to those of skill in the art and can be found in many standard books on molecular 10 protocols. See Sambrook *et al.*, 1989. For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external 15 manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using 20 primer sets designed for optimal sequencing (Pignon *et al.*, 1994). The present invention provides methods by which any or all of these types of analyses may be used. Using the sequences disclosed herein, oligonucleotide primers may be designed to permit the amplification of sequences throughout the STARS genes that may then be analyzed by direct sequencing.

25 (vi) Kit Components

All the essential materials and reagents required for detecting and sequencing STARS and variants thereof may be assembled together in a kit. This generally will comprise preselected primers and probes. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, Sequenase<sup>TM</sup> *etc.*), deoxynucleotides and buffers 30 to provide the necessary reaction mixture for amplification. Such kits also generally will

comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each primer or probe.

### **B. Immunologic Diagnosis**

5       Antibodies of the present invention can be used in characterizing the STARS content of healthy and diseased tissues, through techniques such as ELISAs and Western blotting. This may provide a screen for the presence or absence of cardiomyopathy or serve as a predictor of heart disease.

10      The use of antibodies of the present invention in an ELISA assay is contemplated. For example, anti-STARS antibodies are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a non-specific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of powdered milk. This allows for blocking 15 of non-specific adsorption sites on the immobilizing surface and thus reduces the background caused by non-specific binding of antigen onto the surface.

20      After binding of antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the sample to be tested in a manner conducive to immune complex (antigen/antibody) formation.

25      Following formation of specific immunocomplexes between the test sample and the bound antibody, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for STARS that differs from the first antibody. Appropriate conditions preferably include diluting the sample with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween®. These added agents also tend to assist in the reduction of nonspecific 30 background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures preferably on the order of about 25°C to about 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under 5 conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween®).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-10 sulfonic acid (ABTS) and H<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, e.g., using a visible spectrum spectrophotometer.

The preceding format may be altered by first binding the sample to the assay plate. Then, primary antibody is incubated with the assay plate, followed by detecting of bound primary 15 antibody using a labeled second antibody with specificity for the primary antibody.

The antibody compositions of the present invention will find great use in immunoblot or Western blot analysis. The antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel 20 electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

25

### **C. Treating Defects in STARS Expression or Function**

The present invention also involves, in another embodiment, the treatment of disease states related to the aberrant expression and/or function of STARS. In particular, it is envisioned that STARS activity plays a role in development of cardiac tissue. Thus, increasing levels of 30 STARS, or compensating for mutations that reduce or eliminate the activity of STARS, are believed to provide therapeutic intervention in certain cardiomyopathies.

In addition, by increasing levels of STARS, it is possible that defects in other cardiac genes may be compensated for. STARS may be able to overcome deficiencies in the expression of other cardiac factors.

There also may be situations where one would want to inhibit STARS function or 5 activity, for example, where overexpression or unregulated expression had resulted in cardiac dysfunction. In this case, one would take steps to interfere with or block the expression of STARS, or inhibit its activity.

#### **D. Genetic Based Therapies**

10 One of the therapeutic embodiments contemplated by the present inventors is intervention, at the molecular level, with the events involved in cardiac failure. Specifically, the present inventors intend to provide, to a cardiac cell, an expression construct capable of providing STARS to that cell. The lengthy discussion of expression vectors and the genetic elements employed therein is incorporated into this section by reference. Particularly preferred 15 expression vectors are viral vectors such as adenovirus, adeno-associated virus, herpesvirus, vaccinia virus and retrovirus. Also preferred are liposomally-encapsulated expression vectors.

Those of skill in the art are aware of how to apply gene delivery to *in vivo* situations. For 20 viral vectors, one generally will prepare a viral vector stock. Depending on the kind of virus and the titer attainable, one will deliver  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ ,  $1 \times 10^9$ ,  $1 \times 10^{10}$ ,  $1 \times 10^{11}$  or  $1 \times 10^{12}$  infectious particles to the patient. Similar figures may be extrapolated for liposomal or other non-viral formulations by comparing relative uptake efficiencies. Formulation as a pharmaceutically acceptable composition is discussed below. Various routes are contemplated, including local and systemic, but targeted provision to the heart is preferred. (See, for example Hammond, *et al.*, *supra*, hereby incorporated by reference in its entirety.)

25

#### **E. Combined Therapy**

In many clinical situations, it is advisable to use a combination of distinct therapies. Thus, it is envisioned that, in addition to the therapies described above, one would also wish to provide to the patient more "standard" pharmaceutical cardiac therapies. Examples of standard 30 therapies include so-called "beta blockers", anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, endothelin antagonists, cytokine inhibitors/blockers, calcium

channel blockers, phosphodiesterase inhibitors and angiotensin type 2 antagonists. Also envisioned are combinations with pharmaceuticals identified according to the screening methods described herein.

Combinations may be achieved by contacting cardiac cells with a single composition or 5 pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the agent. Alternatively, the STARS therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and expression construct are applied separately to the cell, one would 10 generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some 15 situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either a STARS gene, protein or 20 STARS agent, or the other agent will be desired. Various combinations may be employed, where STARS is "A" and the other agent is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B  
A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A  
A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

25

Other combinations are contemplated as well.

#### **F. Formulations and Routes for Administration to Patients**

Where clinical applications are contemplated, it will be necessary to prepare 30 pharmaceutical compositions - expression vectors, virus stocks and drugs - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are

essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when 5 recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when 10 administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in 15 therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, 20 nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal, intravascular or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds may also be administered parenterally or intraperitoneally. 25 Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

30 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions

or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, 5 polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, 10 parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the 15 required amount in the appropriate solvent with a variety of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of 20 preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying 25 agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the polypeptides of the present invention may be incorporated 30 with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an

appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a 5 therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, 10 hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage 15 formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, 20 intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035- 25 1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

## V. Drug Formulations and Routes for Administration to Patients

Where clinical applications are contemplated, pharmaceutical compositions will be prepared – *e.g.*, expression vectors, virus stocks and drugs - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free 5 of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector or cells, dissolved or dispersed in a pharmaceutically 10 acceptable carrier or aqueous medium. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes solvents, buffers, solutions, dispersion media, 15 coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into 20 the compositions, provided they do not inactivate the vectors or cells of the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention may be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, 25 intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, as described supra.

The active compounds may also be administered parenterally or intraperitoneally. By way of illustration, solutions of the active compounds as free base or pharmacologically 30 acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene

glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile and fluid to the extent that easy syringability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions may be prepared by incorporating the active compounds in an appropriate amount into a solvent along with any other ingredients (for example as enumerated above) as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the desired other ingredients, *e.g.*, as enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For oral administration the polypeptides of the present invention generally may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution).

Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, 5 abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention generally may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include, for example, acid addition salts (formed with the free amino groups of the protein) derived from inorganic acids (e.g., hydrochloric or phosphoric acids, or from organic acids (e.g., acetic, oxalic, tartaric, mandelic, and the like. Salts 10 formed with the free carboxyl groups of the protein can also be derived from inorganic bases (e.g., sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (e.g., isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions are preferably administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may easily be 15 administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution generally is suitably buffered and the liquid diluent first rendered isotonic for example with sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous and intraperitoneal administration. Preferably, sterile aqueous 20 media are employed as is known to those of skill in the art, particularly in light of the present disclosure. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the 25 condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

## VI. Methods of Making Transgenic Mice

A particular embodiment of the present invention provides transgenic animals that contain a selectable or screenable marker protein under the control of a STARS promoter. Transgenic animals expressing a STARS encoding nucleic acid under the control of an inducible 5 or a constitutive promoter, recombinant cell lines derived from such animals, and transgenic embryos may be useful in determining the exact role that STARS plays in the development and differentiation of cardiomyocytes. Furthermore, this transgenic animal may provide an insight into heart development. The use of constitutively expressed STARS encoding nucleic acid provides a model for over- or unregulated expression. Also, transgenic animals which are 10 "knocked out" for STARS, in one or both alleles, are contemplated.

In a general aspect, a transgenic animal is produced by the integration of a given transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent 4,873,191; which is incorporated herein by reference), Brinster *et al.* (1985); which is 15 incorporated herein by reference in its entirety) and in Hogan *et al.* (1994).

Typically, a gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, 20 mammals, and fish.

DNA clones for microinjection can be prepared by any means known in the art. For example, DNA clones for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer, using standard techniques. The DNA bands are visualized by staining with ethidium 25 bromide, and the band containing the expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a 1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first primed with 3 ml 30 of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind

DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3  $\mu$ g/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA. Other methods 5 for purification of DNA for microinjection are described in Hogan *et al.* (1986), in Palmiter *et al.* (1982); and in Sambrook *et al.* (1989).

In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin 10 (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO<sub>2</sub> asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding 15 cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5 % BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 5% CO<sub>2</sub>, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Randomly cycling adult female mice are paired with vasectomized males. C57BL/6 or Swiss mice or other comparable strains can be used for this purpose. Recipient females are 20 mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5 % avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate 25 buffered saline) and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

## VII. Screening Assays

30 The present invention also contemplates the screening of compounds for various abilities to interact with and/or affect STARS expression or function. Particularly preferred compounds

will be those useful in inhibiting or promoting the actions of STARS in regulating the development and differentiation of cardiomyocytes. In the screening assays of the present invention, the candidate substance may first be screened for basic biochemical activity -- *e.g.*, binding to a target molecule -- and then tested for its ability to inhibit modulate activity, at the 5 cellular, tissue or whole animal level.

**A. Modulators and Assay Formats**

i) Assay Formats

The present invention provides methods of screening for modulators of STARS 10 expression and actin binding activity. In one embodiment, the present invention is directed to a method of:

15 (a) providing an active STARS preparation;  
(b) contacting said STARS preparation with a candidate modulator; and  
(c) measuring the actin binding activity of said STARS preparation,

In yet another embodiment, the assay looks not at actin binding, but at STARS expression. Such methods would comprise, for example:

20 (a) providing a cell in which a STARS promoter directs the expression of a polypeptide;  
(b) contacting said cell with a candidate modulator; and  
(c) measuring the effect of said candidate modulator on said polypeptide,  
25 the polypeptide may be STARS, or it may be an indicator protein.

ii) Candidate Substances

As used herein, the term "candidate substance" refers to any molecule that may potentially modulate STARS expression or function. The candidate substance may be a protein or fragment thereof, a small molecule inhibitor, or even a nucleic acid molecule. The term 5 "candidate modulator" may be used in place of "candidate substance". It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to compounds which interact naturally with STARS. Creating and examining the action of such molecules is known as "rational drug design," and include making predictions relating to the structure of target molecules.

10 The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for a molecule like STARS, and then design a 15 molecule for its ability to interact with STARS. Alternatively, one could design a partially functional fragment of STARS (binding, but no activity), thereby creating a competitive inhibitor. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound or 20 inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of 25 chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small 30 molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (e.g., peptide libraries), is a rapid and efficient way to screen

large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

Candidate compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design STARSting from known inhibitors of hypertrophic response.

Other suitable inhibitors include antisense molecules, ribozymes, and antibodies (including single chain antibodies).

It will, of course, be understood that the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

20

#### **B. *In Vitro* Assays**

A quick, inexpensive and easy assay to run is a binding assay. Binding of a molecule to a target may, in and of itself, be inhibitory, due to steric, allosteric or charge-charge interactions. This can be performed in solution or on a solid phase and can be utilized as a first round screen to rapidly eliminate certain compounds before moving into more sophisticated screening assays. In one embodiment of this kind, the screening of compounds that bind to a STARS molecule or fragment thereof is provided

The target may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the target or the compound may be labeled, thereby permitting determining of binding. In another embodiment, the assay may measure the inhibition of binding of a target to a natural or artificial substrate or binding partner (such as STARS). Competitive

binding assays can be performed in which one of the agents (STARS for example) is labeled. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with the binding moiety's function. One may measure the amount of free label versus bound label to determine binding or inhibition of binding.

5 A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with, for example, STARS, and then washed from the support to remove non-specifically bound protein. Bound polypeptide is detected by various methods.

10 Purified target, such as STARS, can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also, fusion proteins containing a reactive region (preferably a terminal region) may be used to link an active region to a solid phase.

15

#### **C. *In Cytos Assays***

Various cell lines that express STARS can be utilized for screening of candidate substances. For example, cells containing the STARS gene with an engineered indicator can be used to study various functional attributes of candidate compounds. In such assays, the 20 compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell.

Depending on the assay, culture may be required. As discussed above, the cells may then be examined by virtue of a number of different physiologic assays (growth, size,  $Ca^{++}$  effects). Alternatively, molecular analysis may be performed in which the function of STARS and related 25 pathways may be explored. This involves assays such as those for protein expression, enzyme function, substrate utilization, mRNA expression (including differential display of whole cell or polyA RNA) and others.

#### **D. *In Vivo Assays***

30 The present invention particularly contemplates the use of various animal models. Transgenic animals may be created with constructs that permit STARS expression and activity to

be controlled and monitored. The generation of these animals has been described elsewhere in this document.

Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route the could 5 be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood or lymph supply.

10

#### **E. Production of Inhibitors**

In an extension of any of the previously described screening assays, the present invention also provide for methods of producing inhibitors. The methods comprising any of the preceding screening steps followed by an additional step of "producing the candidate substance identified 15 as a modulator of" the screened activity.

### **VII. Examples**

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the 20 examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

25

#### **Example 1 – Materials and Methods**

**Differential cDNA cloning.** For subtractive screening by representational difference analysis (RDA), hearts and the region of the embryo dorsal to the heart, including the neural 30 fold and the first three somites were dissected from E8.25 mouse embryos. Total RNA was isolated from the heart and the dorsal embryonic region using TRIZOL (Gibco). mRNA was

purified using a mRNA purification kit (Amersham Pharmacia) and converted into double-strand cDNA by the Superscript choice system for cDNA synthesis (Gibco). For subtraction screening between heart (Tester) and other embryonic parts (Driver), the RDA method described by Hubank and Schatz 1999 was used. Briefly, the cDNA from both tissues was digested with 5 DpnII and ligated with an R-linker. (The annealed oligonucleotides were: R-Bgl-12: 5'- GATCTGCGGTGA-3' (SEQ ID:NO. 10) and R-Bgl-24: 5'- AGCACTCTCCAGCCTCTACCGCA-3' (SEQ ID:NO. 11) ) Using R-Bgl-24 as primer, cDNA was amplified by PCR and digested with DpnII again. cDNA from the heart was purified by gel extraction and ligated with a J-linker. (The annealed oligonucleotides were: J-Bgl-12: 5'- 10 GATCTGTTCATG-3' (SEQ ID:NO. 12) and J-Bgl-24: 5'- ACCGACGTCGACTATCCATGAACA-3' (SEQ ID:NO. 13)). Tester ligated with J-linker and Driver cDNAs (ratio 1:100) were hybridized at 67°C for 20 hrs. PCR reactions were then performed by primer J-Bgl-24 to yield a PCR product referred to as DPI (Differential product). DPI was digested with DpnII as a new tester and ligated with N-linker (The annealed 15 oligonucleotides were: N-Bgl-12: 5'-GATCTTCCATCG-3' (SEQ ID:NO. 14) and N Bgl-24: 5'- AGGCAACTGTGCTATCCGAGGGAA -3' (SEQ ID:NO. 15)). N-linker-ligated DPI was hybridized with Driver (ratio 1:800) and amplified by PCR two times using N-oligonucleotides as primer. Linkers for tester were replaced with J-oligonucleotides in the third round, and N-oligonucleotides in the fourth round of hybridization. The final PCR products, referred to as 20 DPIV, were cloned into the TA cloning vector (pGEMT easy, Promega) and inserted DPIV fragments were amplified by PCR using N-Bgl-24 as primers to make two identical dot blots. One dot blot was hybridized with tester probe, and the other was hybridized with driver probe to confirm the differential expression of DPIV fragments.

To obtain a full-length STARS cDNA, an E10.5 mouse heart cDNA library (Stratagene) 25 was screened using a 342 bp cDNA fragment isolated by RDA. The longest positive clone contained a 375-amino acid open reading frame, without a stop codon 5' of the first methionine in the sequence. To obtain further 5' sequence, adult mouse heart and skeletal muscle cDNA libraries (Clontech) were screened with a 400 bp cDNA fragment from the 5' end of the longest cDNA as a probe. 5'-RACE was also performed using the SMART 5'-RACE kit (Clontech) and 30 the RLM first choice 5'-RACE kit (Ambion) following the manufacturer's instructions. 5'-

RACE was performed with mRNA from human skeletal muscle (Clontech) and from mouse heart and skeletal muscle. There were no stop codons upstream of the first methionine.

**5 Northern Blot Analysis.** Northern blot analysis was performed using mouse and human multiple tissue Northern blots (Clontech) or total RNA isolated from C2 skeletal muscle cells and mouse hearts by the TRIZOL reagent.

10 C2 skeletal muscle cells were maintained in DMEM with 20% FBS (growth medium) and were induced to differentiate by transfer to medium with 2% horse serum (differentiation medium). Transgenic mice harboring an  $\alpha$ -MHC-calcineurin transgene have been described previously (Molkentin *et al.*, 1998).  $^{32}$ P-labeled probes were prepared from a full-length mouse STARS cDNA and a partial human cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were measured as a control. Hybridizations were performed in Quick Hyb (Clontech).

15 **In situ Hybridization.** RNA probes corresponding to the sense and the anti-sense strands of the STARS cDNA were prepared. *In situ* hybridization was performed on sagittal sections of mouse embryos, as described (Benjamin *et al.*, 1997).

20 **STARS Expression Plasmids.** STARS expression constructs were generated using the pcDNA3.1 mammalian expression vector (Invitrogen) which was modified to contain an amino-terminal Myc-tag. Constitutively active RhoA (L63) and C3 transferase in Prk5 were generously provided by Dr. Alan Hall (University College, London, United Kingdom). The *SM22*-luciferase reporter 1343 bp promoter has been previously described (Li *et al.*, 1995). The smooth muscle  $\alpha$  actin-luciferase reporter contained the 4 kb promoter region ligated into the pBL3basic vector (Promega). The ANF reporter contained the 638 bp promoter region (a generous gift from Dr. 25 Mona Nemer, Universite de Montreal, Montreal, Quebec, Canada). The 4x cfos reporter contained four tandem copies of the cfos CArG box with flanking sequences, as described (Chang *et al.*, 2001). CMV-lacZ (Promega) was used as an internal control for transfection efficiency.

30 **Transient Transfections, Reporter Assays and Immunofluorescence Microscopy.** COS cells were maintained in DMEM with 10% FBS. Eighteen hours after plating, the medium was changed to serum-free medium and transfections were performed at 50% confluence using FuGENE reagent-6 (Roche Molecular Biochemicals) following manufacturer's instructions. Each transfection used 0.15  $\mu$ g of plasmid DNA for the reporter assays, and 0.5-1.0  $\mu$ g

immunohistochemistry. Cytochalasin D 2  $\mu$ M (Sigma-Aldrich), and Y-27632 75  $\mu$ M (Tocris) were added directly just after transfection. Latrunculin B 0.5  $\mu$ M (dissolved in 10% ethanol, Calbiochem) was added 12 hr after transfection and 10% ethanol (final concentration 0.01%) was added to control wells. Cells were harvested 36 hr after transfection and their luciferase and 5  $\beta$ -galactosidase activity were measured.

For measuring immunofluorescence, cells were rinsed with PBS and fixed in 4% paraformaldehyde for 10 min on ice. After rinsing with PBS, COS cells were incubated in 0.1% Triton X-100 in PBS for 5 min on ice. For rat neonatal cardiomyocytes, 1% Triton X-100 in PBS was used. Cells were blocked for 20 min at room temperature in 0.5% BSA in PBS for COS 10 cells and in 1% BSA plus 3% goat serum in PBS for rat cardiomyocytes. Then, cells were incubated with first antibody diluted in 0.5% BSA in PBS for 30-60 min at room temperature or overnight at 4°C. Anti c-myc monoclonal antibody 9E10 (Santa Cruz) was used at a 1:200 dilution. Other antibodies were anti  $\alpha$ -actinin monoclonal antibody (1:400; Sigma-Aldrich),  $\alpha$ -tubulin antibody (1:2000; Sigma-Aldrich) and anti-vimentin antibody (1:500; Sigma-Aldrich). 15 Secondary antibodies were anti mouse /rabbit IgG FITC or Texas red (Vector) used at a 1:200 dilution incubated with or without Phalloidin -TRITC (1:500; Sigma Aldrich).

**Western Blot and Co-immunoprecipitation Assay.** Immunoprecipitation and western blot analysis was performed as described previously (Lu *et al.*, 2000). For immunoprecipitation, cells were incubated with 1 $\mu$ g anti c-myc polyclonal antibody A14 (Santa Cruz). Western blots 20 were performed with anti-actin monoclonal antibody C4 (1:100; Roche Molecular Biochemicals) or anti c-myc monoclonal antibody 9E10 (1:1000; Santa Cruse) followed by incubation with secondary antibody HRP-conjugated anti mouse- IgG (Santa Cruse, Vector). To detect signals, blots were incubated with ECL (Santa Cruz) for 1 min, and exposed to film (Eastman Kodak Co).

**25 Adenoviral transduction and infection to cardiomyocytes.** The full length STARS cDNA including a Kozak consensus sequence was ligated into pACCMViresAdeno (a kind gift from Dr. Robert Gerard) to generate adenovirus expressing STARS and GFP protein by Cre-lox recombination (Aoki *et al.*, 1999). Neonatal rat cardiomyocytes were isolated as described previously (Nicol *et al.*, 2001). After 36 hr, cardiomyocytes were infected for 2 hr with STARS-30 and GFP-expressing adenovirus at a multiplicity of infection of 100. After infection, cells were cultured in non-serum medium (DMEM:Medium199 4:1, penicillin/streptomycin).

**GST-STARS purification and sedimentation assays.** A cDNA encoding the full-length STARS open reading frame was cloned in-frame into vector pGEXKG (Guan and Dixon, 1991). BL21-DE3 cells containing the GST-fusion expression plasmid were grown to an optical density of 0.5 and induced with 1mM IPTG for 3 hr at 370 C. GST-STARS was purified by 5 GST-affinity chromatography using standard techniques. Purified actin and actinin were purchased from Cytoskeleton, Inc. BSA was purchased from Sigma-Aldrich. Actin sedimentation assays were performed as recommended by Cytoskeleton, Inc. Supernatants and pellets were analyzed by SDS-PAGE and coomassie blue staining.

**Antibody production.** To raise an anti-STARS antibody, the full length cDNA was 10 cloned in frame with glutathione-S-transferase in pGEXKG (Guan and Dixon, 1991), which was transformed into BL21 DE3 codon plus (Stratagene). Protein induction and purification was performed following standard protocol. Rabbit immunization was conducted by Cocalico Biologicals. For immunostaining and western blots, IgG was purified from antisera with protein A sepharose beads (Zymed).

15

### Example 2 - Results

**Evolutionary conservation of STARS proteins.** A differential cDNA screen was performed for novel genes expressed in the mouse heart tube at E8.25, but not in other regions of 20 the E8.25 embryo. Positive clones representing heart tube-specific genes were used for whole-mount *in situ* hybridization to confirm their cardiac-specificity. One of the cardiac-specific cDNAs isolated in the screen encoded a novel 375-amino acid protein that is referred to as striated muscle activator of Rho Signalin (STARS), because it is expressed specifically in striated muscle where it binds and bundles actin (see below). The predicted open reading frame 25 of STARS did not contain recognizable protein motifs. Database searches for related genes revealed an apparent human ortholog encoded by a gene located on chromosome 8q23. A partial cDNA encoding a STARS-like protein was also identified in the zebrafish EST database (FIG. 1). No other related genes were identified in searches of the mouse and human genome sequences, indicating that STARS is a single gene without related family members. Genomic 30 sequences with the potential to encode proteins with high homology to the carboxy-terminal 142

amino acids of STARS were also identified in *C. elegans* (F36F2.1, T04A8.4) and *Drosophila melanogaster* (FIG. 1).

5 **STARS is expressed specifically in cardiac and skeletal muscle.** Consistent with the cDNA subtraction scheme, STARS transcripts were detected by *in situ* hybridization only in the primitive heart tube at E8.75 (FIG. 2A). Thereafter, STARS expression was maintained in the heart and was also detected in skeletal muscle after E10.5 (data not shown). Northern analysis of adult tissues revealed three STARS transcripts in mouse heart and skeletal muscle and two transcripts in these human tissues (FIG. 2B). Sequencing of multiple independent cDNAs revealed only a single open reading frame with no evidence for alternative splicing within the 10 protein coding region. Therefore, it is believed that the multiple transcripts reflect the presence of different 3' -untranslated sequences.

15 In the C2 skeletal muscle cell line, STARS expression was undetectable in proliferating myoblasts, but was upregulated after three days in differentiation medium, following the formation of myotubes (FIG. 2C). Thus, STARS appears to be a relatively late marker for skeletal muscle differentiation.

20 STARS was examined to determine if it was regulated during hypertrophic growth of the heart, which is associated with up-regulation of a specific set of cardiac genes. Expression of activated calcineurin in the heart results in profound hypertrophy that ultimately progresses to dilated cardiomyopathy (Molkentin *et al.*, 1998). STARS expression was upregulated dramatically in calcineurin transgenic mice at 3 months of age, when hearts showed extreme concentric hypertrophy without obvious left ventricular failure (FIG. 2D). By 6 months of age, when hearts from these mice had dilated and were in late stages of failure, STARS expression was further up-regulated. Cardiac expression of STARS was also up-regulated following banding of the thoracic aorta, which results in hypertrophy due to pressure-overload.

25 **STARS localization in cardiomyocytes.** Western blot analysis of adult mouse heart extracts an anti-STARS antibody revealed a single protein species of 45 kD, similar to the predicted size of STARS (FIG. 3A). This band comigrated with a Myc-tagged STARS protein expressed in transfected COS cells.

30 To determine the subcellular distribution of STARS, rat primary cardiomyocytes were immunostained with the STARS antibody (FIG. 3B, a&d). STARS staining showed a periodicity reminiscent of sarcomeric localization. The sarcomeric localization of STARS abuts the Z-line

on both sides as demonstrated by a partial overlap with  $\alpha$ -actinin (FIG. 3B, b&c, e&f). This periodicity demonstrates that STARS is localized to the I-band of the sarcomere. In addition to I-band localization, a portion of STARS localizes to sarcomeric structures between Z-lines (FIG. 3B, c&f). A schematic of STARS localization is depicted in FIG. 3C. Together, these results  
5 indicated that STARS functions as a muscle-specific actin-binding protein.

**Mapping the F-actin binding domain of STARS.** To further investigate the function of STARS, the subcellular localization of a Myc-tagged STARS protein in transiently transfected COS cells was examined. STARS strongly colocalized with F-actin in stress fibers and membrane ruffles, which were also stained by Phalloidin-TRITC (FIG. 4A; c, d and e). STARS-transfected cells also exhibited an increased number of actin stress fibers and especially prominent thick fibers, indicating that ectopic STARS expression induced stress fiber formation and bundling (FIG. 4A; f, g and h). Other structural proteins, including tubulin, vimentin, and keratin did not colocalize with STARS (data not shown).

To map the region of STARS that mediated the association with F-actin, immunofluorescence was performed with a series of STARS deletion mutants in transfected COS cells. Deletion mutants lacking residues from the carboxy-terminus into the conserved region of STARS (mutants N346, N323, N304, N279, N233, N100, and N101-233), and a deletion mutant within the conserved region (C96), did not affect the association with F-actin, but abolished the ability of STARS to bundle actin stress fibers (FIG. 4A; l, m, n and 5, and data  
15 not shown). In contrast to C96, a deletion mutant that encoded only the conserved region (C142) not only associated with F-actin, but also increased stress fibers, although bundled stress fibers were not as frequent as seen with the wild-type protein (FIG. 4A; i, j and, k). Deletion mutant Del234-279 was able to associate with F-actin, but lost the ability to bundle actin fibers. Smaller  
20 sub-deletions of this region retained the ability to bundle actin, indicating that the amino acid residues responsible for this function were distributed across residues 234-279. These findings indicated that the conserved carboxy-terminal domain of STARS was responsible for actin-bundling and that there were multiple, non-overlapping regions of the protein capable of weakly  
25 associating with F-actin *in vivo*.

**Co-immunoprecipitation of STARS with actin.** The actin binding function of STARS  
30 was further investigated by co-immunoprecipitation experiments in COS cells transiently transfected with Myc-tagged proteins. As shown in FIG. 4B, the wild-type protein and the

conserved carboxy terminal region (C142) co-immunoprecipitated with actin. Deletions that removed the carboxy-terminal portion of the conserved region did not bind actin (mutants N346, N323, N304, N279, N233, N100, and N101-233), whereas small internal deletion mutants within the conserved region (Del 234-247, Del 248-262, Del 263-279) retained weak actin-binding 5 activity in this assay. The larger deletion mutant in this region (Del 234-279) failed to co-immunoprecipitate with actin. These results revealed a direct correlation between actin binding/bundling activity *in vivo* and co-immunoprecipitation of STARS with actin (FIG. 5).

**STARS stimulates SRF-dependent transcription via actin polymerization.** Recent 10 studies have shown that stabilization of the actin cytoskeleton stimulates the transcriptional activity of SRF (Mack *et al.*, 2001), but the proteins involved in this phenomenon have not been identified. To examine whether STARS might participate in a signaling pathway between the cytoskeleton and the nucleus, the promoter of the *SM22* gene, which is regulated by SRF in muscle cells (Li *et al.*, 1997; Kim *et al.*, 1997), was tested to determine if it was responsive to 15 STARS. Remarkably, STARS stimulated the expression of an *SM22* luciferase reporter by 40-fold in transiently transfected COS cells (FIG. 6A). Mutation of the CArG boxes in the *SM22* promoter abolished responsiveness to STARS, demonstrating the involvement of SRF in this response. In F9 cells, which have low endogenous SRF expression (Li *et al.*, 1997), STARS had a minimal effect on the *SM22* promoter (FIG. 6A), further indicating that the effects of STARS 20 were mediated by SRF. Stimulation of SRF activity is not a general property of actin-binding proteins, as  $\alpha$ -actinin has no effect on SRF activity (data not shown). STARS also weakly stimulated the  *$\alpha$ -smooth muscle actin* promoter, which is regulated by SRF (Mack and Owens, 1999), and it activated an *E1b* promoter linked to four tandem copies of the CArG box and flanking sequences from the *c-fos* promoter (FIG. 6B). However, it did not stimulate the *atrial natriuretic factor (ANF)* promoter (FIG. 6B) which is also regulated by SRF (Hines *et al.*, 1999). 25 It also did not activate the *cytomegalovirus* promoter (data not shown).

A summary of the properties of STARS deletion mutants is shown in FIG. 5. Comparison of the complete set of STARS deletion mutants showed a direct correlation between the ability to bind actin and bundle actin fibers and to stimulate *SM22* transcription (FIG. 6C). The conserved carboxy terminal region of STARS was necessary and sufficient to stimulate SRF 30 activity, as demonstrated by the finding that mutant C142 was as effective as the full-length protein in activating *SM22*-luciferase.

**Effects of actin depolymerizing agents on *SM22* promoter activation by STARS.**

To examine whether STARS stimulates SRF activity via its effects on actin dynamics, COS cells were treated with cytochalasin D (CD), which prevents actin polymerization (Sampath and Pollard, 1991), and latrunculin B (LB), which sequesters monomeric actin (Morton *et al.*, 2000).

5 In STARS-transfected cells, only short fragments of disrupted stress fibers were observed in the presence of 2  $\mu$ M CD or 0.5  $\mu$ M LB (data not shown). CD itself increased *SM22* promoter activity by 8-fold, consistent with previous results (Sotiropoulos *et al.*, 1999; Mack *et al.*, 2001). STARS increased promoter activity 18-fold in the presence of CD. In contrast, LB treatment abolished *SM22*-luciferase activity even in cells transfected with STARS (FIG. 6D). These 10 findings indicated that STARS might stimulate SRF activity by reducing the cellular pool of G-actin as a result of its ability to enhance actin polymerization. The opposing effects of CD and LB are likely to reflect their differential effects on the pool of G-actin; whereas CD dimerizes G-actin (Goddette and Frieden, 1986), LB sequesters G-actin (Morton *et al.*, 2000).

**Involvement of RhoA in SRF activation by STARS.** In light of the ability of RhoA to

15 stimulate SRF activity by promoting actin polymerization (Mack *et al.*, 2001; Sotiropoulos *et al.*, 1999), the effects of a constitutively active RhoA mutant (L63) and STARS on *SM22* promoter activity were compared. As shown in FIG. 6E, STARS and RhoA L63 activated the *SM22* promoter to comparable levels and together stimulated activity to higher levels.

20 Rho signaling is inhibited by the Rho kinase inhibitor Y-27632, which inhibits stress fiber formation (Maekawa *et al.*, 1999), and C3 transferase, which specifically ADP-ribosylates RhoA (Nemoto *et al.*, 1992). Treatment of COS cells with Y-27632 (75  $\mu$ M) or transfection with a C3 expression plasmid reduced the stimulatory activity of STARS on the *SM22* promoter by 55% and 88%, respectively (FIG. 6F). Together, these results indicate that STARS requires a basal level of RhoA activity to enhance SRF activity.

25

**Example 3 - Discussion**

**Stimulation of SRF-dependent transcription by STARS.**

30 Previous studies indicated that G-actin suppresses the activity of SRF (Sotiropoulos *et al.*, 1999), and several observations indicate that STARS activates SRF by relieving this repressive influence. For example, agents such as latrunculin B, which sequesters G-actin monomers (Morton *et al.*,

2000), inhibit the activity of STARS. The ability of STARS to stimulate actin polymerization and cross-linking would also be expected to reduce the G-actin pool. It is important to point out that the stimulation of actin polymerization *per se* is not sufficient to account for the effects of STARS on SRF activity, because STARS is able to increase SRF activity in the presence of 5 cytochalasin D, which prevents actin polymerization and also stimulates SRF (Sotiropoulos *et al.*, 1999; Mack *et al.*, 2001).

A working model of a potential mechanism of action of STARS within the context of the regulation of actin dynamics is shown in FIG. 7. Actin treadmilling determines the relative distribution of the monomeric G-actin and polymerized F-actin states. Previous studies 10 suggested that G-actin suppresses the activity of SRF (Sotiropoulos *et al.*, 1999). The results suggest that STARS activates SRF by relieving this repressive influence, as a result of its ability to stimulate actin polymerization and thereby reduce the G-actin pool. Such a mechanism would account for the ability of latrunculin B, which sequesters G-actin monomers (Morton *et al.*, 2000), to interfere with the activity of STARS.

15 While polymerization of actin by STARS appears to be coupled to SRF activation, stimulation of actin polymerization *per se* is not sufficient to account for the effects of STARS on SRF activity, because STARS is able to increase SRF activity in the presence of cytochalasin D, which prevents actin polymerization by binding to the plus-end of F-actin, where it blocks further addition of actin subunits. Cytochalasin D also stimulates SRF activity alone, 20 presumably by reducing the level of free G-actin monomers (Sotiropoulos *et al.*, 1999; Mack *et al.*, 2001).

In principle, G-actin could inhibit SRF directly or it could sequester cofactors required 25 for SRF activation (Sotiropoulos *et al.*, 1999). In light of the actin-binding properties of STARS, it may stimulate SRF by acting as a sink to sequester G-actin and thereby relieve repression on SRF. G-actin has been shown to shuttle to the nucleus (Wada *et al.*, 1998) and also to be contained within the SWI-SNF chromatin remodeling complex (Van Etten *et al.*, 1994; Zhao *et al.*, 1998; Rando *et al.*, 2000). Whether STARS might alter the incorporation of actin into this complex is a possibility. Since STARS is localized to the cytoplasm, it may not stimulate transcription by associating directly with SRF or other transcriptional components.

30 SRF activates muscle-specific transcription by recruiting myogenic transcription factors, such as GATA4, Nkx2.5 and myocardin (Chen and Schwartz, 1996; Belaguli *et al.*, 2000; Morin

*et al.*, 2001; Wang *et al.*, 2001). Although STARS is a muscle-specific protein, its ability to stimulate SRF activity in nonmuscle cells indicates that it does not require these myogenic transcription factors for activity. STARS is upregulated after the onset of myocyte differentiation and may act as an actin cross-linker during myofibrillogenesis, thereby further 5 enhancing SRF activity in differentiated muscle cells and reinforcing the expression of SRF-dependent sarcomeric genes.

**Regulation of STARS activity, SRF activity and muscle gene expression by Rho signaling.** Rho signaling promotes the formation of F-actin and could deplete the G-actin pool. Sotiropoulos concluded that the effects of Rho on SRF are secondary to its effects on the 10 cytoskeleton and are mediated by a decrease in G-actin as a result of actin polymerization (Sotiropoulos *et al.*, 1999). Stimulation of SRF activity by STARS appears to require at least a basal level of RhoA signaling, as demonstrated by the ability of the Rho kinase inhibitor Y-27632 and C3 transferase to diminish SRF activation by STARS. Conversely, constitutively active RhoA enhances the stimulatory effect of STARS on SRF activity. The fact that Y-27632 15 only partially inhibits STARS activity may indicate the involvement of other Rho effectors in the mechanism for STARS action. It is also possible that these inhibitors interfere with the SRF-activating properties of STARS through an indirect mechanism due to changes in cell morphology.

Rho signaling plays an important role in muscle gene expression. Previous studies have 20 shown that RhoA activates the skeletal muscle  $\alpha$ -actin promoter, which is SRF-dependent (Wei *et al.*, 1998; Wei *et al.*, 2001). Activation of RhoA by Gq-coupled receptor agonists also induces premyofibrils, myofibril organization and ANF expression in primary cardiomyocytes (Thorburn *et al.*, 1997; Aoki *et al.*, 1998; Hoshijima *et al.*, 1998). STARS could not activate the ANF promoter, although RhoA can upregulate ANF expression in cardiomyocytes. This 25 indicates that STARS does not activate Rho directly. Of course, stimulation of SRF activity by Rho does not require STARS, since Rho can activate SRF in nonmuscle cells in which STARS is not expressed. In contrast, STARS requires basal Rho activity to activate SRF, because the Rho inhibitor C3 and the Rho kinase inhibitor Y27632 decreased SM22 promoter activity significantly, indicating that STARS might have cross-talk or interaction with some effector 30 molecules of Rho and to Rho kinase. Taken together, the results indicate that stimulation of SRF

activity by STARS activation is not mediated by the same effector-molecules that mediate *ANF* promoter activation by RhoA in cardiomyocytes.

**Linking the cytoskeleton and the sarcomere to muscle gene expression.** STARS expression is maintained in adult cardiac and skeletal muscle and is dramatically up-regulated 5 during hypertrophic growth of the heart in response to calcineurin activation and pressure-overload, which is associated with calcineurin activation (Leinwand, 2001). Sarcomere organization is a hallmark of cardiac hypertrophy. During hypertrophic growth of cardiomyocytes, STARS might organize new myofibrils, whereas extreme overexpression might result in disorganization of actin bundles with resulting cardiac dysfunction as observed in 10 failing hearts.

The integrity of the cytoskeleton and sarcomere has a profound influence on gene expression and growth of muscle cells. The actin bundling/binding and SRF-activating properties of STARS provide a potential link between myocyte structure and the program for muscle gene expression.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the 20 compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may 25 be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## IX. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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U.S. Patent 5,792,453  
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U.S. Patent 4,196,265

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